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Th1-Type Cytokines Production Is Decreased in Kidney Transplant Recipients With Active Cytomegalovirus Infection

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Cytomegalovirus (CMV) infection is a major complication after kidney transplantation. Despite antiviral therapy the infection contributes significantly to high morbidity. The present study was aimed at determining: (a) the stimulation index (S.I.) of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) and (b) the levels of Th1- and Th2- related cytokines in kidney transplant recipients with and without active CMV infection. Thirty-five patients with, and 44 without active CMV infections, as diagnosed by a CMV antigenemia assay, were inducted into this study. After PHA stimulation of PBMC from patients, stimulation index (S.I.) was determined by radioactive thymidine uptake while the production of Th1-type cytokines (interleukin-2 [IL-2], interferon-γ [IFN-γ], and tumor necrosis factor-α [TNF-α]) and Th2-type cytokines (IL-4, IL-10) were measured by enzyme-linked immunosorbent assay. PBMC of patients with active CMV infection showed significantly lower S.I. values than patients without an ongoing CMV infection ($P < .0001$). Levels of Th2-type cytokines in CMV-infected and uninfected kidney recipients were similar; however, the levels of the Th1-type cytokines were significantly lower in CMV-infected patients. Low levels of Th1-type cytokines seem to correlate well with active CMV infection in kidney recipients. ***J. Med. Virol. 60:223–229, 2000.***

KEY WORDS: viral infection; immunosuppression; T helper cells

INTRODUCTION

Although cytomegalovirus (CMV) infection in immunocompetent individuals usually causes only mild or subclinical disease, in immunocompromised patients it may cause severe clinical symptoms [Meyers et al., 1986]. However, the mechanisms of tissue injury caused by CMV infection in renal transplant recipients are not well understood. Studies in immunosuppressed humans and animals have revealed that $CD4^+$ and $CD8^+$ T cells are of crucial importance in the maintenance of immunity to CMV as well as for the eradication of an ongoing infection [Borysiewicz et al., 1988; Jonjic et al., 1994]. In human and animal experiments, the adoptive transfer of syngeneic, polyclonal $CD8^+$ T cells to immunosuppressed individuals has been shown to provide protection from CMV disease [Quinnan et al., 1982; Reusser et al., 1991]. Adoptive immunotherapy with CMV-specific $CD8^+$ T cell clones from allogeneic donors has been shown to prevent the development of CMV disease [Reddehase et al., 1985, 1987]. In addition, allogeneic transfer of CMV-specific $CD8^+$ cell clones has been found to reconstitute cellular immunity against CMV in vivo [Riddell et al., 1992]. It has also been shown that infusion of increasing doses of cells results in an increased CMV-specific cytotoxic T lymphocyte response in recipients [Walter et al., 1995].

It is clear that the Th1 and Th2 subsets are of major importance in determining the class of immunoprotective function in infectious diseases. Thus a bias either toward Th1-dominance or Th2-dominance can make a difference between effective elimination of the virus and exacerbation of the disease [Mosmann and Sad, 1996]. Research on HIV infection [Clerici and Shearer, 1993], murine AIDS [Gazzineli et al., 1992], vaccinia virus [Actor et al., 1993], herpes simplex virus [Jayaraman et al., 1993] and influenza virus [Graham et al., 1994] has shown that generally Th2 responses exacerbate the infection, whereas Th1 responses are protective. Given the strong influence exerted by Th1- and Th2-type immunity on the outcome of infections, we

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considered it of importance to elucidate Th1- and Th2-type profiles in CMV infection in renal transplantees.

Because the proliferative response of T lymphocytes to mitogens generally reflects the status of cellular immunity, we have measured the stimulation index (S.I.) values of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from kidney recipients with and without active CMV infection. Culture supernatants of PHA-stimulated PBMC were analyzed for Th1-type and Th2-type cytokines, to determine the status of Th1-Th2 profiles.

MATERIALS AND METHODS

Study Population

Seventy-nine kidney transplant patients (31 women, 48 men) who received kidney transplants during 1996 and 1997 were included in this study. Their ages ranged from 18 to 60 years; most subjects were 30–45 years old (median age = 35 years). All patients received an immunosuppressive regimen of cyclosporin A, azathioprine, and steroids. Of the 35 patients positive for the CMV-pp65 antigen, 32 had one or more of the following symptoms associated with CMV infection/disease: fever, arthralgia, leukopenia, thrombocytopenia, pneumonitis, hepatitis, retinitis, and gastrointestinal ulceration. Diagnosis of CMV infection/disease conformed to the guidelines suggested in the Workshop on CMV Disease [Ljungman and Plotkin, 1995]. Patients in this study had to have at least one of the CMV-associated symptoms in addition to being antigenemia-positive to be considered as having CMV disease. Serial samples were collected at different intervals after transplantation and patients were monitored for 6 months. Blood samples were collected in two ethylenediamine tetraacetic acid (EDTA)-containing tubes and transferred to the laboratory within 2–3 hr. Antigen detection and mitogen-induced activation of PBMC were carried out on the same day.

CMV Antigenemia Assay (AA)

A total of 5 ml blood was collected from each patient and processed immediately. Leukocytes were isolated by the dextran sedimentation method. Following incubation and centrifugation, the cell pellet was suspended in phosphate-buffered saline (PBS) and the erythrocytes were lysed with 0.8 mM ammonium chloride. The remaining cells were centrifuged, washed in PBS, counted and spotted onto glass slides (50,000 cells per spot). They were then dried and fixed in acetone-methanol, stained with CMV-vue Kit (Incstar, Inc., Stillwater, MN) according to the manufacturer's recommended procedure for immunoperoxidase staining. Numbers of cells containing the CMV-specific pp65 antigen were counted under a light microscope. Patients with $\geq$5 cells containing the CMV-specific pp65 antigen out of 50,000 cells were considered to be positive for AA. Each patient was tested three or more times by the AA at different time points. Patients who tested positive at the time of mitogen-induced stimulation were considered AA-positive in this study.

Mitogen-Induced Stimulation of PBMC

PBMC from 35 antigenemia-positive and 44 antigenemia-negative transplant recipients were obtained by Ficoll-Hypaque (Pharmacia Biotech, Sweden) density gradient centrifugation of peripheral blood at 1500 × *g* for 20 min. PBMC were suspended in RPMI-1640 medium (GIBCO BRL, Gaithersburg, MD) containing 10% fetal calf serum, aliquoted into 96-well tissue culture plates at a density of 10^5 cells per well, and then stimulated with PHA at a concentration of 5 μg/ml for a period of 96 hr. The concentration of 5 μg/ml was chosen on the basis of optimal proliferation in our laboratory. The supernatants from some of the wells were harvested 24 and 96 hr later, while some of the wells were pulsed with [^{3}H] thymidine (1 μCi per well) at 72 hr for assessment of mitogen-induced proliferation. Thymidine-pulsed wells were harvested 18 hr later and the radioactivity estimated. The S.I. was calculated as a ratio of thymidine uptake by PHA-stimulated cells to that by nonstimulated cells. Background thymidine uptake in the absence of PHA was in the range of 80–500 cpm. Samples yielding S.I. of less than 50 were considered as proliferation-negative. The cut-off of 50 was based on experience in our Clinical Immunology Laboratory that an S.I. less than 50 generally indicates low proliferation due to poor immune status of the subjects.

Assay for Cytokines

Levels of the Th1-type cytokines, interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ), and the Th2-type cytokines, IL-4 and IL-10, were estimated in the supernatants of PBMC of 44 CMV antigenemia-negative kidney recipients and 35 antigenemia-positive recipients stimulated with PHA for 24 (for IL-2) and 96 hr (for TNF-α, IFN-γ, IL-4, and IL-10). Th1-type cytokines and Th2-type cytokines were assayed by enzyme-linked immunosorbent assay (ELISA) using kits obtained from Immunotech SA (France). These consisted of sandwich type ELISA; briefly, the first step led to the capture of the relevant cytokines by monoclonal anti-cytokine antibodies bound to the wells of microtiter plates. In the second step, biotinylated monoclonal antibody was added together with streptavidin-enzyme (peroxidase or alkaline phosphatase) conjugate. The biotinylated antibody binds to the antibody-antigen complex, and in turn, binds the conjugate. After incubation, the wells were washed and the binding of streptavidin-enzyme via biotin was followed by the addition of a chromogenic substrate. The intensity of the coloration produced is proportional to the concentration of the cytokine present in the sample. The sensitivity of each of the assays was as follows: 5 pg/ml for IL-2, 10 pg/ml for TNF-α, 3 pg/ml for IFN-γ, 5 pg/ml for IL-4, and 5 pg/ml for IL-10.

Statistical Analysis

The standard Mann–Whitney test was used for nonparametric comparisons of median cytokine levels.

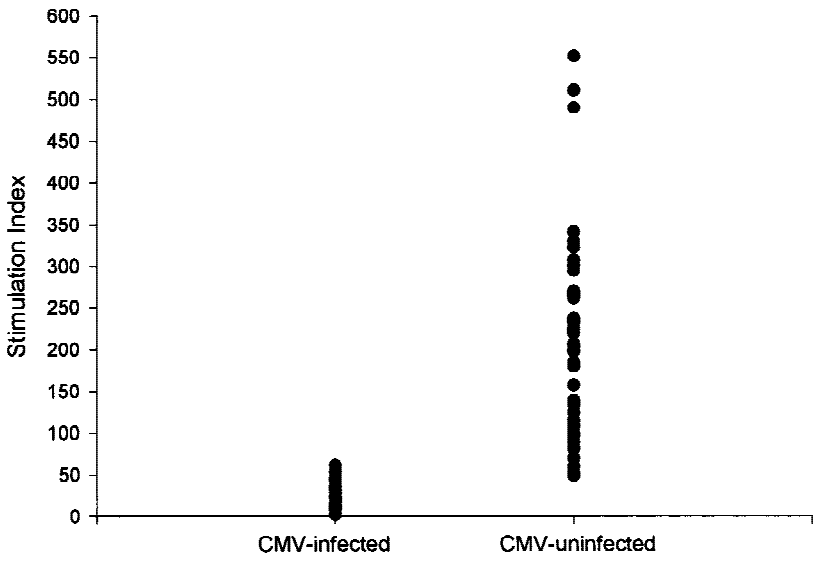

Fig. 1. Stimulation indices (S.I.) of 35 cytomegalovirus (CMV)-infected and 44 CMV-uninfected kidney recipients.

RESULTS

PHA-Induced PBMC Proliferation

Figure 1 shows the stimulation indices of PBMC from antigenemia-positive and antigenemia-negative kidney transplant recipients after stimulation with PHA. It was found that the indices in the antigenemia-positive kidney recipients was inversely related to the degree of antigen positivity ($r^2 = 0.052$, Fig. 2); in other words, patients who were highly antigenemia positive had lower stimulation indices than were patients who had low antigenemia. In the group of antigenemia-positive recipients, the number of CMV-pp65-positive cells varied between 10 and 500 and the S.I. was as low as 2 when the number of CMV-pp65-positive cells was 500; the S.I. was as high as 50 when the number of CMV-pp65-positive cells was around 10. As can be seen from Table I, of the 35 antigenemia-positive patients 33 (94%) were proliferation negative. On the other hand, of the 44 antigenemia-negative patients only 1 was unresponsive to PHA (2%). Figure 3 shows the stimulation indices of PBMC from antigenemia-positive kidney recipients before the detection of active CMV infection, during active CMV infection, and after the recovery from active CMV infection. It was found that the indices in the antigenemia-positive kidney recipients were decreased during active CMV infection (mean S.I. = 24, $P < .0005$) when compared with the indices in the same kidney recipients before active CMV infection (mean S.I. = 80, $P < .0005$). It was also found that the indices in these recipients are marginally increased, but still low after loss of antigenemia (mean S.I. = 28, $P < .05$).

Th1 Cytokines

The levels of IL-2, TNF-α, and IFN-γ were consistently higher in kidney recipients without an active CMV infection than in the group of recipients with an active CMV infection (Fig. 4). These differences were statistically significant in the case of IL-2 ($P < .05$) at 24 hr, and IFN-γ ($P < .0005$) and TNF-α ($P < .005$) at 96 hr. P values were calculated by the Mann–Whitney test conducted on median cytokine levels. The median pg/ml values (range) for IFN-γ, TNF-α, and IL-2 in the antigenemia-positive patients are 7 (0–321), 311 (0–6893.9), and 447 (0–5136), respectively; on the other hand, in the antigenemia-negative patients, the median pg/ml values are 131 (0–815.6), 2213 (0–7074), and 1501 (0–7696), respectively. It is evident that kidney recipients with an active CMV infection produce lower levels of Th1 cytokines than do the kidney recipients without an active CMV infection. Therefore, these data suggest that CMV infection impairs the production of Th1-related cytokines.

Th2 Cytokines

The levels of IL-4 and IL-10 were measured in antigen-positive and antigen-negative patients. There were no statistically significant differences in the levels of IL-4 and IL-10 between antigenemia-positive and -negative groups (Fig. 5).

Th1/Th2 Cytokines Ratios

The ratio of Th1 to Th2 cytokines in a given sample is considered to be of greater significance than the levels of cytokines alone. Therefore, we calculated the ratios of different Th1 to Th2 cytokines produced by PHA-stimulated PBMC. The mean values of Th1 cytokines were compared with that of Th2 cytokines produced by PBMC of kidney recipients with and without active CMV infections. The difference between the two groups in some of the Th1:Th2 ratios was found to be striking. Table II depicts the ratios of Th1 to Th2 cytokines for the two time points tested. For example, the IL-2:IL-4 ratio at 24 hr and TNF-α:IL-4 ratio at 96 hr were lower in the antigenemia-positive group as compared with the antigenemia-negative group, indicating a lower Th1-bias in the antigenemia-positive group. In some other cases the differences in ratios were not as striking, even though the ratios were consistently lower in

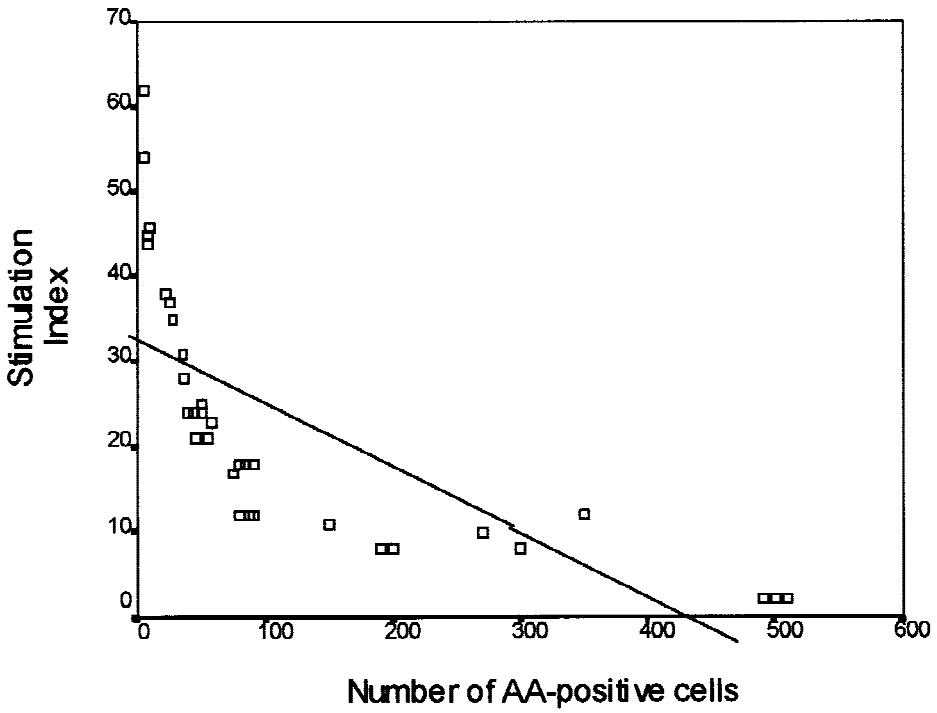

Fig. 2. Correlation between lymphocyte proliferation (stimulation index) in response to mitogenic stimulation and the number of antigenemia assay (AA)-positive cells in kidney transplant recipients.

TABLE I. Mitogen-Induced Proliferation* of PBMC in CMV-Infected and -Uninfected Transplant Recipients

	Proliferation positive	Proliferation negative	Total
CMV infected	2	33	35
CMV uninfected	43	1	44
Total	45	34	79

PBMC, peripheral blood mononuclear cells; CMV, cytomegalovirus; S.I., stimulation index.
*PBMC proliferation was considered positive when the S.I. $\geq$ 50.

infected individuals. Thus, general trend supports a lower production of Th1 cytokines by the antigenemia-positive group than by the antigenemia-negative group.

DISCUSSION

For the prevention of an active CMV infection and CMV disease, cellular immunity in general, and the action of cytotoxic T cells to CMV-infected target cells in particular, is the most important factor in the host defense mechanisms [Rook et al., 1984; De Waal Malefyte et al., 1993]. In kidney recipients the outcome of CMV infection depends to a great extent on the activity of T cells [Mosmann and Coffman, 1989], and therefore PBMC proliferation to PHA was used as an indicator of the T-cell responses in kidney transplant recipients with and without CMV infections.

Results of PHA-induced proliferative responses of PBMC revealed that recipients with an active CMV infection had lower levels of proliferation (S.I. < 50) as compared with recipients without active CMV infections (S.I. $\geq$ 50). These results show that the reduction in the PHA-induced response of PBMC correlates well with the development of an active CMV infection. This finding illustrates the importance of helper-T-cell responses, and fits in with the well-recognized role of CMV-specific cytotoxic T cells in recovery from opportunistic CMV infections [Quinnan et al., 1982; Reusser et al., 1991]. On the other hand, the diminished T-cell response may be due to the immunosuppressive effect of active CMV infection in kidney transplant recipients [Rook et al., 1984]. The data (Fig. 3) indicate that stimulation indices are higher before CMV infection and declines with the detection of CMV infection. Thus it appears likely that CMV infection leads to immunosuppression, reflected here as a decline in the T-cell response to mitogenic stimulation. Interestingly, this immunosuppression is also reflected by a decline in the production of Th1-type cytokines.

$CD4^+$ T cells regulate both cellular and humoral immune responses in vivo, primarily by releasing numerous cytokines [Heinzel et al., 1991; Sad and Mosmann, 1994]. $CD4^+$ T cells exist as at least two distinct subsets, the Th1 and Th2. Th1 cells are characterized by the production of high levels of IFN-γ, TNF-β, and IL-2, whereas Th2 cells preferentially release IL-4, IL-5, IL-10, and IL-13 [Mosmann and Coffman, 1989; Romagnani, 1992]. Th1-type cytokines are generally associated with effective defense mechanisms against intracellular infectious agents by activating macrophages; promoting granuloma formation and inducing delayed type hypersensitivity (DTH) reactions. Th2-associated cytokines such as IL-4 and IL-10 sometimes allow the progression of intracellular infectious diseases by down-regulating Th1 responses [Heinzel et al., 1991; Sad and Mosmann, 1994]. It should be pointed out that

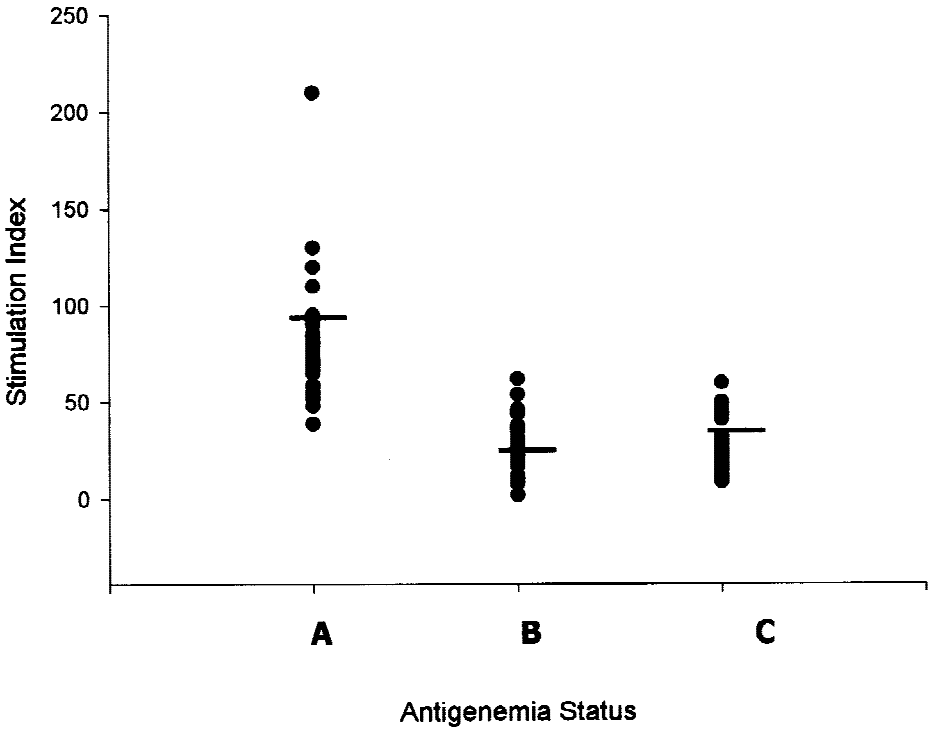

Fig. 3. Stimulation indices in **(A)** kidney transplantees before cytomegalovirus (CMV) infection as tested by the antigenemia assay, **(B)** during infection, and **(C)** after infection as indicated by a negative antigenemia assay.

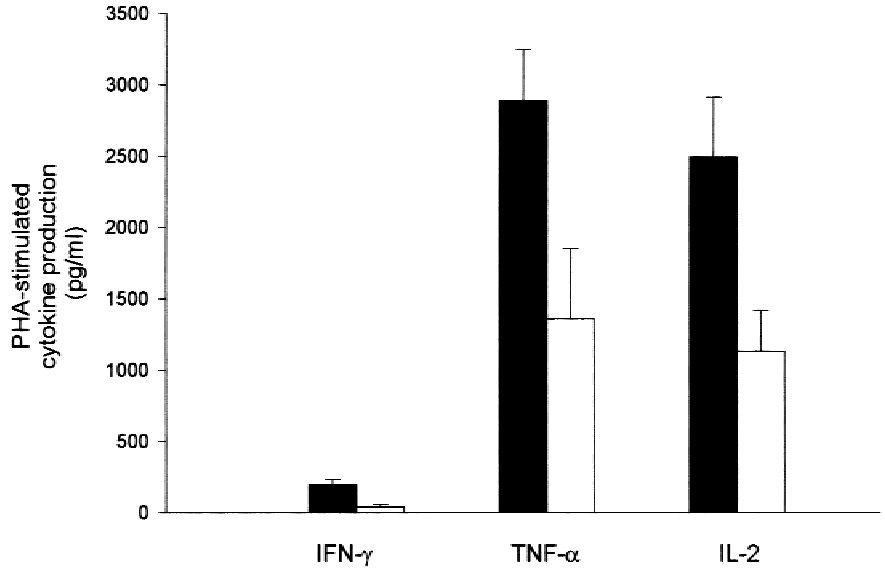

Fig. 4. Levels of Th1-type cytokines produced by mitogen-induced peripheral blood mononuclear cells (PBMC) after 24 hr of culture for interleukin-2 (IL-2) and 96 hr of culture for interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α). Dark bars represent mean levels (±SEM) of the cytokines in cytomegalovirus (CMV)-negative individuals; white bars represent mean levels in CMV-positive subjects.

whereas both Th1 and Th2 cells produce TNF-α, it is secreted at higher levels in Th1 responses [Romagnani, 1992] and that it mediates cytotoxic activity as a part of cell-mediated immunity. TNF-α also elicits the production of IFN-γ, a Th1-type cytokine. Thus, although TNF-α is not a characteristic Th1-type cytokine, it is of great significance in Th1-type cell-mediated responses.

This study shows that Th1 cytokine levels elicited in response to stimulation with a mitogen are lower in kidney recipients with active CMV infections than in those without active CMV infections. Because Th1 cells provide help for cell-mediated reactions against viral infection, a decline in Th1 activity may well lead to poor defenses against viruses. Furthermore, a decline in Th1-type activity may adversely affect the outcome of adoptive immunotherapeutic transfer of virus-specific T cells. Studies on the adoptive transfer of CMV-specific clones, for example, are currently underway [Riddell and Greenberg, 1997]; the present data on depressed Th1-type activity may be relevant in this regard. There appears to be no significant difference in Th2 cytokine secretion between CMV-positive and CMV-negative patients. Similar observations have been published [Sparrelid et al., 1997] in relation to

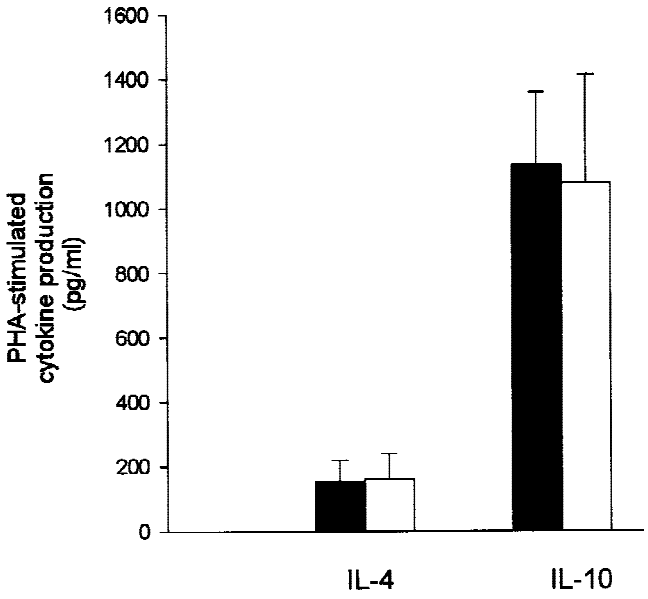

Fig. 5. Levels of Th2-type cytokines secreted by mitogen-induced peripheral blood mononuclear cells (PBMC) after 96 hr of culture. Dark bars represent mean levels (±SEM) of the cytokines in cytomegalovirus (CMV)-negative individuals; white bars represent mean levels in CMV-positive subjects.

TABLE II. Th1:Th2 Cytokine Ratios in CMV-Infected and -Uninfected Transplant Recipients*

Th1:Th2 ratio	CMV uninfected	CMV infected
After 24 hr of culture		
IL-2:IL-4	16.0	7.0
IL-2:IL-10	2.2	1.1
After 96 hr of culture		
TNF:IL-4	18.6	8.4
TNF:IL-10	2.6	1.3
IFN:IL-4	1.3	0.2
IFN:IL-10	0.1	0.04

*CMV, cytomegalovirus; IL, interleukin; TNF, tumor necrosis factor; IFN, interferon.

bone marrow transplantation; recipients with active CMV infections had a low level of Th1 cytokine production whereas Th2 cytokine production remained unaltered. Though that study dealt with local production of Th1 and Th2 cytokines within the lung, the results are similar to our findings.

It is possible that the correlation between CMV infection and down-regulation of Th1 cytokines is actually due to immunosuppressive therapy. However, this explanation is unlikely, because both the CMV-infected and uninfected individuals received the same immunosuppressive regimen. It is more likely that CMV infection itself has a selective suppressive effect on Th1-type reactivity [Boland et al., 1990]. Because cell-mediated immunity in general, and Th1-type immunity in particular, is important for protection against CMV infection, the balance between Th1-related and Th2-related cytokines may determine the outcome of a CMV infection.

The data presented above indicate the importance of Th1-type cytokines in the pathogenesis of CMV infection.

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